FUNGAL PHYSIOLOGICAL RESPONSE TO TEMPERATURE AND NITROGEN AVAILABILITY

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SHANA WHITNEY

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This thesis was examined and approved in partial fulfillment of the requirements for the degree of Master of Science in Natural Resources by:

Thesis Director, Dr. Serita Frey, Professor of Natural Resources & the Environment

Dr. Stuart Grandy, Associate Professor of Natural Resources & the Environment

Dr. Kevin Geyer, Postdoctoral Researcher of Natural Resources & the Environment

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Approval signatures are on file with the University of New Hampshire Graduate School.

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ABSTRACT

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Shana Whitney

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Microbial carbon use efficiency (CUE; the proportion of assimilated C allocated towards biomass production) is a powerful regulator of global soil C stocks, and there is a need to integrate accurate and dynamic CUE parameters into C-cycling models. However, there is uncertainty in the CUE-response to warming and increased N deposition and how this response may vary among groups of microbes. Here, we conducted a laboratory incubation of ten phylogenetically diverse fungal isolates grown in liquid culture to evaluate potential environmental and physiological controls over CUE. Isolates were grown at 15ºC or 25ºC and under low or high N concentrations. We measured respiration rates, growth rates, CUE, and a suite of potential extracellular enzyme activities. We show that CUE was consistently reduced at elevated temperatures due to increased respiration and decreased growth rates. Additionally, we show that overall N addition did not significantly impact respiration, growth, or CUE. However, there were N addition effects that were group- and species-specific. Further, we show that there are tradeoffs between physiological traits, such as growth rate and resource acquisition, that result in distinct growth strategies among phylogenetic groups. Our work demonstrates that there are significant environmental and physiological controls over microbial CUE and that CUE is similar and predictable among distinct phylogenetic groups of fungi.

1. Introduction

Soils represent the largest pool of Earth's terrestrial carbon (C) and release substantial amounts of CO_2 to the atmosphere annually. Specifically, soils hold over three times the amount of atmospheric C and emit 60-75 Pg of CO_2-C each year, which is about six times more than anthropogenic emissions (Schlesinger and Andrews 2000, Pan *et al.,* 2011; Trivedi *et al.,* 2013). Small changes in microbial processes such as decomposition and the subsequent stabilization of microbial products can have disproportionate effects on ecosystem C cycling (Six *et al.,* 2006; Frey *et al.,* 2013). Thus, integrating accurate and dynamic parameters of key microbial processes would improve global C models, especially under global climate change (Wieder *et al.,* 2015).

Microbial carbon use efficiency (CUE), defined as the portion of assimilated C a microbe incorporates into biomass (Manzoni *et al.,* 2012; Geyer *et al.,* 2016), is considered one such critical parameter. Our understanding of environmental and physiological influences over CUE is poorly constrained, however, making it difficult to parameterize these models. For example, soil microbes experience vast environmental heterogeneity, including shifts in soil temperature and nutrient availability, that potentially influence CUE. Many studies have assessed microbial CUE under varying environmental conditions and have shown that CUE response is strongly variable depending on conditions (both natural and experimental) that microbes experience (Manzoni *et al.,* 2012). Additionally, groups of soil microbes have diverse life-history and growth strategies, which often leads to generalizations of microbial groups based on physiological traits in global C models (Bardgett *et al.,* 2008; Wieder *et al.,* 2013; Allison, 2014). For example, microbial groups are often defined as either efficient or inefficient and are often not parameterized with differential responses to environmental shifts. It is uncertain if these generalizations are

adequately representative of microbial diversity and how environmental variations may control them (Rousk and Frey, 2015; Wieder *et al.,* 2015). Due to these uncertainties, it is crucial to further investigate environmental and physiological controls on CUE and understand whether the effects of these controls are species dependent.

Rising temperatures and increased nitrogen (N) deposition are expected to be particularly important controls on CUE. It is theoretically accepted that the CUE of heterotrophic microbes (including fungi) will decrease with warming, primarily attributed to increased catabolic processes relative to increased anabolic processes, where C will be used for protein turnover and cell repair rather than biosynthesis (Manzoni *et al.,* 2012; Sinsabaugh *et al.,* 2013; Bradford 2013). The observed CUE response to warming has been variable, where CUE has been shown to decrease, increase, or have no response (Devevre and Horwath, 2000; Steinweg *et al.,* 2008; Lehmeir *et al.,* 2016; Dijkstra *et al.*, 2011; Frey *et al.,* 2013; Öquist *et al.,* 2017; Li *et al.,* 2019). Some of this variability is due to methodological differences, such as in techniques to measure microbial respiration and growth, environmental conditions, and study duration. A portion of this variability, however, is likely accredited to underlying differences in microbial communities.

Increased rates of atmospheric N deposition can directly or indirectly influence physiological activity of soil microbes. Increased soil N has been shown to consistently suppress decomposition and activity of lignin-degrading enzymes, resulting in an accumulation of soil C (Carreiro *et al.,* 2000; Knorr *et al.,* 2005; Zak *et al.,* 2011; Frey *et al.,* 2014). It is unclear whether this slowed decomposition is caused by shifts in microbial community composition towards microbes less able to degrade recalcitrant C sources, or by direct effects of elevated N on microbial physiology and stoichiometric requirements (Mooshammer *et al.,* 2014; van Diepen

et al., 2017; Morrison *et al.,* 2018). It is predicted that there is a hump-shaped relationship between resource C:N and microbial CUE, where microbial CUE is highest at an optimal C:N where energy and nutrients are balanced (Frost *et al.*, 2006; Sinsabaugh *et al.,* 2016). CUE will increase as a substrate approaches optimal C:N because microbes will be more able to maintain their stoichiometric requirements, whereas CUE will decrease once a substrate is elevated above optimal C:N because microbes will respire excess C due to N limitation (Mooshammer *et al.,* 2014; Manzoni *et al.,* 2012, 2017). This relationship is particularly important for soil fungi, who are primary decomposers of complex and N-poor substrates (Schneider *et al.,* 2012). Studies show that addition of inorganic N generally increases respiration and growth rates of microbial communities (Koranda *et al.,* 2014) and of fungal isolates (Boberg *et al.,* 2008; Keiblinger *et al.,* 2010). However, these studies also show that increased N has inconsistent effects across microbial species, suggesting that CUE response to increased N may be variably important among a variety of microbial communities. Additionally, there are few studies that look at both temperature and N effects on CUE at a population scale, so potential confounding effects of these factors remain uncertain (Savage *et al.,* 2013; Eberwein *et al.,* 2015).

Soil fungi, which are sensitive to environmental fluctuations, are extremely diverse in their growth strategies and their functional capacity to decompose organic matter. Three phylogenetic groups prevalent in soil communities are the Basidiomycota, Ascomycota, and Mucoromycotina. Many species within Basidiomycota are white-rot fungi and can decompose recalcitrant C sources. Additionally, Basidiomycota species are generally thought to be more efficient and competitive in nutrient limited environments (Treseder and Lennon 2015). The Ascomycota species can decompose complex C sources as well but are limited in that they

cannot decompose lignin. The Ascomycota generally have relatively intermediate growth in soil and have the genetic capacity to tolerate stressful conditions (Morrison 2017, 2018). Lastly, the Mucoromycotina species are often only able to use labile C sources. The Mucoromycotina are also thought to be more inefficient, fast-growing species that favor a resource-enriched environment. Although these generalizations are often applicable for species within each phylum, there are certainly exceptions. However, because these three phyla are distinct enough from one another and are representative of most soil fungi, it's important to understand how the physiology of species within each grouping is influenced by warming and elevated N.

Understanding the CUE response to a changing environment at population scales is important because CUE is an indicator of metabolism, so it is directly related to organismal physiology, which is sensitive to abiotic conditions. Soil warming and N deposition will directly affect organismal physiology through biosynthesis rates and cellular maintenance based on stoichiometry (Geyer *et al.,* 2016). Thus, understanding how CUE is regulated across species under different environments is an important component of predicting how community-level CUE will respond as microbial community composition shifts.

It is hypothesized that there are inherent tradeoffs between CUE and other traits, such as growth rates and investment in enzyme production (Pfeiffer *et al.,* 2001). For example, higher growth efficiency is generally coupled with lower growth rates (i.e. the rate-yield tradeoff; Pfeiffer *et al.,* 2001, Allison 2014; Lipson, 2015). In current trait-based C-cycling models, this tradeoff is generally represented by copiotrophic and oligotrophic strategies, where copiotrophs grow rapidly when resources are abundant and are less efficient due to higher energetic costs for cellular maintenance; whereas, oligotrophs grow slowly and are specialized to grow efficiently in

resource-poor environments (Koch, 2001; Lipson *et al.* 2009; Roller and Schmidt 2015). Empirical evidence of this theoretical tradeoff is quite limited (Keiblinger *et al.,* 2010; Roller and Schmidt, 2015). This tradeoff between growth rate and CUE is also thought to be coupled with a tradeoff between CUE and resource acquisition, where investment in extracellular enzymes to obtain more resources comes at the cost of reduced efficiency (Allison 2014; Malik *et al.,* 2019). These physiological tradeoffs are likely controlled to some extent by environment. For example, the tradeoff between CUE and investment in enzymes may not exist when substrates are plentiful. These tradeoffs offer a convenient way to bin different species of fungi based on genetic capacities such as growth strategy and potential for decomposition of organic materials (Treseder and Lennon, 2015; Morrison 2017). Different functional groups of fungi will vary in sensitivity to environmental stressors, and thus form distinct feedbacks on ecosystem-scale proceses. For example, as soil temperatures and resource availability change, different groups of fungi will dominate the community based on their sensitivity to environmental shifts, and their growth strategies will have ecosystem-level C-storage implications. Understanding how microbial groups regulate their CUE will help predict overall CUE changes at the community-level when community composition shifts.

How would we best incorporate different functional groups of fungi into ecosystem-C models? There are current trait-based models that allow for unique functional groups of microbial communities such as the DEMENT model and the MIMICS model (Allison, 2014; Wieder *et al.*, 2015). However, more empirical evidence is needed to support that the generalizations in these models are representative enough of the diverse growth strategies across microbes. Other frameworks that offer more specific levels of groupings should also be

considered when modelling microbial traits based on differential groups. For example, two distinct frameworks are the competitor-stress tolerator-ruderal adaptive strategy framework (CSR), and the high yield-resource acquisition-stress tolerance life history framework (YAS; Grime, 1977; Morrison 2017; Malik *et al.,* 2019). These frameworks are based on the physiological tradeoffs discussed above. Groupings based on phylogenetic relatedness is another feasible option to represent microbial diversity because many functional traits are phylogenetically conserved, making it possible to define different growth strategies across phyla (Lennon *et al.,* 2012; Amend *et al.,* 2015; Morrison 2017).

Here we conducted an incubation experiment to measure CUE and enzyme activity of ten fungal species grown under varying temperature and N availability conditions. Our primary objectives were to determine (1) the response of fungal CUE and enzyme activities to temperature and N availability, (2) whether there are tradeoffs between fungal CUE and growth rate or enzyme activity, and (3) whether species within distinct phylogenetic groupings have similar growth rates, CUE, and enzyme activities and responses to environmental conditions. We hypothesized that CUE would decrease with increased temperature because respiration would increase more rapidly than growth rate. We also predicted that CUE would decrease under N limitation because the scarcity in N would divert C utilization to either investment in enzymes or to overflow respiration. Additionally, we predicted that there would be negative relationships between CUE and growth rate and CUE and enzyme activity. Finally, we hypothesized that environmental and physiological controls over CUE would be predictable and similar among phylogenetic groups of fungi.

2. Materials and methods

We conducted a three-factor incubation experiment of ten fungal isolates grown in liquid media under varying temperatures and N availabilities. Fungal respiration rates, growth rates, and CUE were measured at 15ºC or 25ºC and at low (C:N = 123:1) or high N (C:N = 20:1) availability. Extracellular enzyme activities were also assessed. A complete growth curve was replicated three times for each experimental combination (10 species x 2 temperatures x 2 N levels x 3 replicates = 120 growth curves)

2.1 Culture and incubation conditions

Ten saprotrophic fungal species were grown in a liquid media to measure CUE response under different environmental treatments. The selected species were originally isolated from decomposing leaf litter and soil collected from the Harvard Forest Long-term Ecological Research (LTER) site in Petersham, MA (described by van Diepen *et al.,* 2017). Cultures were stored on potato dextrose agar (PDA) slants at 4ºC. The criteria by which the isolates were selected are described in Morrison (2017). The ten isolates were selected as they are highly abundant in the fungal community present on decomposing leaf litter at Harvard Forest, represent a broad phylogenetic range (i.e., four Basidiomycota, four Ascomycota, two Mucoromycotina), and represent different decomposer functional groups (Table S3). Additionally, these isolates have been used in previous and on-going experiments, which allowed us to compare and compile datasets. Species identification was confirmed using the Basic Local Alignment Search Tool (BLAST, Alstchul *et al.,* 1990) to compare ITS sequences to the NCBI nucleotide database.

Isolates were subsampled from long-term storage slants onto PDA plates with similar chemical composition to the liquid media used later (i.e., low or high N). This allowed the isolates to acclimate to low or high N conditions and eliminated repeated sampling from storage slants. Prior to experiment initiation, each isolate was transferred to a new PDA plate of the same chemical composition and was grown again at either 15ºC or 25ºC for at least five days to allow the isolate to acclimate to the target experimental temperature (Crowther and Bradford, 2013).

Experiments were conducted in liquid culture, with isolates grown in a sterilized modified potato dextrose broth medium. The use of a liquid culture medium was favorable because (1) it allowed for direct measurements of biomass production, rather than relying on measurements that are correlated to growth rate, such as hyphal extension rates or isotopically labeled substrate incorporation, and (2) it was a reductionist approach that allowed us to define traits of individual groups of fungi, rather than traits of a whole community (Geyer *et al.,* 2016; Crowther *et al.,* 2018). Culture medium contained, per L, 12.6 g D-glucose (0.42 M C), 1.4 g potato infusion extract (46.6 mM C, 3.8 mM N; Fisher 52424), and 8.53 g MES buffer (40 mM). pH was adjusted to 4.5 using potassium hydroxide. We utilized two N levels that were chosen based on threshold element ratios with an average biomass C:N of 10:1, where a 123:1 medium was expected to induce N limitation and a 20:1 medium was expected to alleviate N limitation (Manzoni *et al.,* 2012; Sinsabaugh *et al.,* 2016). Ammonium nitrate (0.78 g; 19.5 mM N) was added to the medium for the high N treatment. This medium was partitioned into 160 mL gas-tight vials (100 mL media per vial) and autoclaved for 20 minutes at 121ºC, after which it was inoculated with the individual isolates by cutting an ~8-mm^3 plug from the growing edge of the culture plate and submerging it into the liquid media. Vials were then loosely capped with

aluminum foil to ensure sterile conditions while allowing air transfer. Vials were incubated in the dark at either 15ºC or 25ºC and shaken continuously at 75 rpm to maintain homogeneity of the media and aerobic conditions. Incubation duration varied for each species (3-28 d) depending on growth rate. Eight vials were prepared for each growth curve to ensure the full growth curve was measured.

2.2 Measurement of carbon use efficiency

Respiration and growth rates were measured for each species and treatment combination (15ºC or 25ºC; 123:1 or 20:1 C:N) at least four times during log-phase growth. At regular time intervals during growth, vials were capped and sealed using gas-tight septa, flushed with CO_2-free air for 10 minutes at 15 PSI to eliminate headspace CO_2, and returned to the target temperature for 6 to 24 hours depending on the respiration rate of the species. The headspace was then sampled for CO_2 concentration, measured using a LI-6252 Infrared Gas Analyzer (LI-COR, Inc., Lincoln, NE). Ambient CO_2 was accounted for by measuring the CO_2 concentration of a control vial (sterile liquid media treated the same as sample vials). The entire culture was then filtered through a GF/C glass filter under vacuum in order to collect biomass. Three washes of 100 mL filtered DI water were conducted to ensure no liquid media remained on the biomass. Liquid media was collected and frozen at -20ºC prior to these washes for subsequent extracellular enzyme analysis. Biomass was then collected from the filter, frozen at -80ºC, freeze-dried, and weighed. Biomass-C was determined by grinding freeze-dried biomass and combusting in a Perkins-Elmer CHN Series II 2400 Elemental Analyzer (Perkin Elmer Inc., Waltham, MA).

Carbon use efficiency was calculated as $\frac{mass\ specific\ growth\ rate\ (\mu)}{(\mu + mass\ specific\ respiration\ rate\ (Rmass))}$ during log-phase growth. Measurement of CUE during this portion of the growth curve effectively measures intrinsic CUE, which is assumed to be the maximum CUE achievable by that species under present growth conditions and is often used as a parameter in ecosystem models (Sinsabaugh *et al.,* 2013; Geyer *et al.,* 2016). Growth rate (μ) was calculated as biomass-C produced biomass-C^{-1} hr^{-1} by normalizing for biomass C content of each species and using the slope of the linear portion of the log-transformed growth curve. Specific respiration (R_{mass}) was calculated in the same way that μ was calculated, by regressing CO_2-C produced hr^{-1} over biomass-C.

2.3 Extracellular enzyme activities

Extracellular enzyme activities were evaluated on the remaining liquid media collected after biomass filtration as adapted from Saiya-Cork *et al.* (2002), DeForest (2009), and Allison (2009). Liquid media was thawed to room temperature and mixed prior to enzyme assays. Thawed media (200 μl) and enzyme-specific substrates (50 μl) were pipetted into 96-well microplates. We assessed five enzymes that are involved in decomposition of C, N, and phosphorus substrates: β-d-glucosidase (BG), N-acetyl-ß-glucosaminidase (NAG), phosphatase (PHOS), phenol oxidase (ABTS), and peroxidase (TMB). Phenol oxidase and peroxidase were measured using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), and 3,3',5,5' Tetramethylbenzidine, respectively. Hydrolytic enzymes BG, NAG, and PHOS were measured using the methylumbelliferyl-(MUB) linked substrates β-D-glucopyranoside, N-acetyl-ß-D-glucosaminide, and phosphate, respectively. Microplates were incubated at 22ºC for 15 min to 4 hrs, which represented the substrate-specific time necessary to achieve maximum activity. After

the appropriate incubation period, net fluorescence was measured for hydrolytic enzymes at an excitation wavelength of 360 nm and an emission wavelength of 450 nm, and absorbance was measured for oxidative enzymes at 420 nm (ABTS) or 450 nm (TMB) using a BioTek Synergy^{TM}HT Multi-detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT). All enzyme assays were conducted on liquid media collected from the log-phase of growth to capture potential enzyme activity at the same time that CUE was calculated. Final enzyme activity values were calculated using formulas described in DeForest (2009), with final units as nmol substrate h^{-1} mg dry biomass^{-1}.

2.4 Statistical analyses

Three-way analysis of variance (ANOVA) was used to test whether temperature, N availability, or fungal phylum had a significant effect on respiration rate, growth rate, CUE, or enzyme activities. When treatment effects were significant, Tukey's *post-hoc* test was used. Data that violated assumptions of normality were square-root transformed. Data that violated the assumption of homoscedasticity were analyzed using mean comparisons using a linear mixed effects model, which allows for heterogeneous variance structure (Pinheiro *et al.,* 2015). All univariate analyses were conducted in R 3.4.1 (R Core Team, 2017).

Principal components analysis (PCA) was used to extract a set of composite variables that described the variance in the enzyme activity dataset. A variance-covariance matrix was used in the PCA because data were log-transformed prior to the analysis to meet assumptions of linear relationships and normal distribution. A randomization test was used to determine the number of principal components to interpret. To confirm the statistical significance of *a priori* groups in the PCA ordination, we conducted a multiresponse permutation procedure (MRPP).

We then investigated the relationships between significant principal component axes and growth rate, respiration rate, and CUE. All multivariate analyses were conducted in PC-ORD (MjM Software).

3. Results

3.1 Effect of temperature, N availability, and phylum on physiological traits of fungal isolates

Across all species, temperature and phylum significantly affected respiration, growth, and CUE, while N availability had no effect. Phylum had the strongest effect on respiration and growth, while temperature had the strongest effect on CUE (Table 1). The only significant interaction term was the temperature x phylum respiration response.

On average, CUE decreased by 15% from 15ºC to 25ºC ($P < 0.0001$; Fig. 1c). However, the magnitude of decline in CUE with warming was variable across species, and there was one case where CUE increased significantly with warming, which indicates that CUE-temperature response may be species-specific (Fig. S2a). Respiration generally increased and growth rate generally decreased with warming, suggesting that 25ºC was above an ideal temperature range for each species and likely induced stress ($P < 0.0001$ and $P = 0.012$, respectively; Fig.1a,b).

Overall, there was no effect of increased N availability on respiration, growth, or CUE (Fig 1d-f). However, the Basidiomycota had a significant increase in CUE with increased N ($P = 0.048$; Table S1; Fig. S2b), due to a decrease in respiration rate (Table 1; Fig. S1a), suggesting that the Basidiomycota are more sensitive to resource availability than the Ascomycota or Mucoromycotina. Additionally, there were species-specific responses to increased N. At 15ºC, CUE decreased significantly for two species, increased significantly for two species, and did not

change for six species under elevated N (Fig. S2b). At 25ºC, CUE decreased significantly for four species, and increased significantly for six species with elevated N (Fig. S2b). These responses were visually stronger at 25ºC, although there was no statistically significant interaction between temperature and N.

Respiration and growth were significantly different across phyla, where the Basidiomycota species had the lowest respiration and growth, the Ascomycota species had intermediate respiration and growth, and the Mucoromycotina species had relatively high respiration and growth (Fig. 1g,h). This resulted in the Mucoromycotina with the lowest CUE averaging 0.50 (± 0.10), the Ascomycota with an intermediate CUE averaging 0.65 (± 0.15), and the Basidiomycota with the highest CUE averaging 0.75 (± 0.15; Fig. 1i). Carbon use efficiency was not significantly different between the Ascomycota and Basidiomycota, although we suspect that any significant difference in intrinsic CUE was diluted by divergent responses to the environmental treatments. For example, the Basidiomycota had a significant increase in CUE with elevated N, while the Ascomycota had no significant CUE response to elevated N. This response would increase the Basidiomycota species' CUE closer to what we might expect to see for the Ascomycota species' CUE.

All enzyme activities (BG, PHOS, NAG, ABTS, TMB) were significantly different among phyla (Fig. S4). The Basidiomycota species had the highest activity of BG, NAG, ABTS, and TMB. The Ascomycota species had the highest activity of PHOS. The Mucoromycotina species had very low enzyme activity across all enzymes. Both the Ascomycota and Mucoromycotina species had very little or no oxidative enzyme activity. Across all species, there were variable effects of temperature and N availability on enzymes: BG and PHOS were not

significantly impacted by either temperature or N availability and NAG activity was higher with high N availability ($P < 0.0001$). Effects of temperature and N availability on oxidative enzymes were only examined for the Basidiomycota; ABTS and TMB were significantly lower at high N availability, and TMB was significantly higher at 25ºC ($P < 0.0001$, $P = 0.0512$, $P = 0.037$, respectively). Principal components analysis of potential enzyme activities revealed that the full enzyme profiles grouped by phylum, which were shown to be significantly distinct from one another through pairwise MRPP comparisons ($P < 0.0001$; Fig. S5).

3.2 Tradeoffs between growth, CUE, and resource acquisition

There was a significant tradeoff between growth rate and CUE when looking across all species. Within each phylum, however, there were positive relationships between growth rate and CUE. There were significant negative correlations between resource acquisition and respiration and growth rate, while there was a hump-shaped relationship between resource acquisition and CUE.

There was a significant correlation between growth rate and CUE (Fig. 2). There was a significant inverse correlation when looking at this relationship overall at 15ºC ($r^2 = 0.19$, $P = 0.0005$; Fig 2a) and at 25ºC ($r^2 = 0.11$, $P = 0.01$; Fig. 2a). However, when looking at this relationship within each phylum, there is a positive correlation between growth rate and CUE for the Basidiomycota ($r^2 = 0.12$, $P = 0.016$), Ascomycota ($r^2 = 0.28$, $P = 0.0001$), and Mucoromycotina ($r^2 = 0.32$, $P = 0.004$; Fig. 2b). There was no clear influence of temperature or N availability on these relationships (Fig. 2). Although insignificant, there was also a positive trend between the response of CUE and growth rate to temperature (i.e. Q_{10}; Fig. S3a), where species with larger temperature-driven increases in growth rates had smaller declines in CUE.

Additionally, there was a significant inverse relationship between Q_{10} of CUE and Q_{10} of respiration rates ($r^2 = 0.62$, $P < 0.0001$; Fig. S3b), where larger temperature-driven increases in respiration rates had larger declines in CUE.

There was a strong tradeoff between resource acquisition and respiration and growth rates. PCA Axis 1 of enzyme activities was strongly correlated with NAG, ABTS, and TMB activities and represented 43% of the variation in the data (Fig. S5). So, we chose to use this axis to represent investment in resource acquisition to test for potential relationships with respiration, growth, and CUE. Resource acquisition was strongly correlated with respiration ($r^2 = 0.53$, $P < 0.0001$; Fig. 3a) and growth rates ($r^2 = 0.52$, $P < 0.0001$; Fig. 3b), where Basidiomycota species had relatively low respiration and growth rates and relatively high potential for enzyme activity, the Ascomycota had intermediate respiration, growth rates and potential for enzyme activity, and the Mucoromycotina had relatively high respiration and growth rates and relatively low potential for enzyme activity. There was a significant quadratic relationship between resource acquisition and CUE ($r^2 = 0.24$, $P < 0.0001$; Fig. 3c), where the Mucoromycotina and Ascomycota had a positive relationship between CUE and resource acquisition, while the Basidiomycota had a negative relationship between CUE and resource acquisition.

4. Discussion

We show that fungal respiration, growth, and CUE are strongly affected by temperature and that effects of N availability are species-specific. Additionally, we show that there is an overall tradeoff between growth and efficiency, although that tradeoff is reversed within phylogenetic groups of fungi. Further, we show that there is a tradeoff between investment in

resource acquisition and respiration and growth, while there is a hump-shaped relationship between investment in resource acquisition and CUE. Lastly, we demonstrate that phylogenetically distinct groups of fungi have similar growth strategies and responses to temperature and N availability.

4.1 Environmental controls on CUE and enzyme activities

Warming from 15ºC to 25ºC significantly increased respiration and decreased growth and CUE. These results are consistent with previous studies that have demonstrated a decline in CUE of microbial communities (Devevre and Horwath, 2000; Steinweg *et al.,* 2008) and of individual species (Lehmeir *et al.*, 2016; Morrison 2017) with warming. This has important implications because warming will have direct effects on microbial CUE at the population scale by influencing physiology, regardless of indirect effects on CUE due to shifts in community composition (Cheng *et al.*, 2017; Morrison *et al.,* 2019). Our results indicate that while community shifts are likely to occur with warming, we can still expect intrinsic CUE of that community to decrease because warming has a consistent negative effect on CUE at the population scale. Of course, since these results stem from a short-term laboratory incubation, we were not able to see potential long-term acclimation or other responses that could occur under long-term warming. For example, the Harvard Forest soil warming experiments offer an opportunity to observe decadal effects of warming on CUE and other soil microbial community dynamics. Studies based on these experiments have shown that warming induces increased soil respiration rates initially, but eventually respiration rates decline due to depletion of labile C, declines in microbial biomass, and shifts in microbial CUE (Bradford *et al.,* 2008; Frey *et al.,* 2013; Melillo *et al.,* 2017). Additionally, previous studies have examined the effect of warming

on CUE when microbes are utilizing multiple substrates ranging in lability (Frey *et al.,* 2013; Oquist *et al.,* 2017; Soares and Rousk 2019) These studies demonstrate that when microbes utilize more complex substrates, CUE is often lower and the interaction between warming and increasing substrate complexity is variable. Thus, because our study only looked at CUE in a simple substrate media, we only can infer how CUE may be affected by warming in a non-limiting scenario (i.e. intrinsic CUE).

Additions of inorganic N did not significantly influence respiration, growth, or CUE across species. This result was not entirely unexpected, as other studies have shown limited fungal CUE response to increased N. Keiblinger *et al.* (2010) found that when growing two Ascomycota species under different N availabilities, one species' CUE was positively correlated with resource C:N, while the other species' CUE was not affected by increased N; whereas, bacterial CUE was consistently higher with increased N in that study. This supports the finding that fungi are more C-limited than bacteria and are less sensitive to elevated N (Six *et al.,* 2006). Our results of insensitive CUE response to increased N could be explained by a hypothesis offered by Manzoni and Porporato (2009): under nutrient limitation, two scenarios can occur. First, uptake of both C and the limiting nutrient (N) is inhibited, resulting in no impact on CUE. Alternatively, excess C is taken up that cannot be put towards biomass production, so overflow respiration occurs and CUE decreases. For species that had insensitive CUE to increased N, we found that biomass was not significantly lower at low N (Fig. S7), so these species potentially were not experiencing N limitation. Additionally, most of the species grown here that had insensitive CUE responses have high genetic capacity for inorganic N uptake, potentially making

them less sensitive to fluctuations in N availability (Manzoni *et al.*, 2012; Treseder and Lennon, 2015; Morrison 2017).

A closer look at the effect on N availability on microbial CUE reveals that the CUE of Basidiomycota did increase with higher N availability (Table S1; Fig. S2). This response was consistent across three out of four Basidiomycota species tested, which happen to be the species with highest potential for lignin degradation (*Gymnopus sp., G. dryophilus,* and *P. stipticus)*. For these same three species, this response was coupled with a significant decrease in oxidative enzymes with inorganic N addition (Fig. S6) which is consistent with previous studies looking at the effect of long-term soil N enrichment on lignin-degrading enzyme activities (Carreiro *et al.,* 2000; van Diepen *et al.,* 2015; Morrison *et al.,* 2018). Our finding that even in the absence of complex substrates, ligninolytic enzymes are still repressed with increased inorganic N, is supported by other literature (Fog 1988; Carreiro *et al.*, 2000), which suggests that inorganic N has a direct effect on enzyme production. Additionally, because oxidative enzyme production was higher at low N concentrations, more C was also respired (although insignificant) because investment in enzyme activities is often paired with higher respiration due to high energetic costs (Moorhead *et al.,* 2012). This result was consistent with a similar study examining Basidiomycota (Lashermes *et al.* 2016). The result of N deposition decreasing enzymatic activity of lignin-degrading fungi has particular community-level implications because Basidiomycota species will be less able to utilize recalcitrant C sources which will cause longer C residence times in soil (Carreiro *et al.,* 2000). It has been shown that long-term N deposition shifts microbial communities towards more stress-tolerant fungi (Treseder and Lennon, 2015; Zhong *et al.*, 2015; Morrison *et al.,* 2018). Because community composition is expected to shift with N

deposition and elevated N has significant physiological effects at the species-level, it is particularly important to understand physiological controls and tradeoffs of functional groups that are selected for under N deposition (i.e. stress tolerators).

4.2 Tradeoffs between growth, CUE, and resource acquisition

We demonstrate that, when looking across ten representative fungal taxa from a forest soil, there is a negative relationship between growth rate and CUE. This finding is supported by many theoretical and empirical studies examining the rate-yield tradeoff (Pfeiffer *et al.*, 2001; Six *et al.*, 2006; Allison 2014; Blagodatskaya *et al.*, 2014; Lipson *et al.*, 2009, 2015; Treseder and Lennon 2015), which show that communities with higher growth rates generally have lower CUE. This tradeoff is generally explained by a community shift from K to r-strategists. Additionally, the slope of this relationship is not temperature dependent, which suggests that although warming will lower microbial efficiency overall, warming may not be a strong control on tradeoffs among physiological traits.

While there was an overall tradeoff between growth and CUE, there was a positive relationship when looking within phylogenetic groups. This positive relationship has been well documented, where aquatic- and culture-based studies demonstrate that taxonomically similar species have a positive relationship between growth and CUE (Russel and Cook 1995; Pirt 1965; del Giorgio and Cole 1998; Babel *et al.*, 2009; Fonte *et al.*, 2013). These positive relationships in our data well-illustrate the unique growth strategies of each group of fungi. For example, each slope of the regression line for each phylum is very different. Mucoromycotina has a slope closer to 0, suggesting that these species have a high range of growth rates, but their CUE is much more constrained. Contrastingly, Basidiomycota has a slope closer to 1, suggesting that these species'

CUE is quite sensitive whereas their growth rate is relatively constrained. These positive relationships suggest additional risks associated with shifts in community composition. For example, if the environment selects for an oligotrophic community (i.e. Basidiomycetes), overall CUE may become more sensitive to environmental stressors (i.e. CUE range: 0.38-0.96. On contrast, if the environment selects for a copiotrophic community, overall CUE may be less sensitive (i.e. CUE range: 0.32-0.67).

In addition to relationships observed between growth and CUE, we show that there is a tradeoff between investment in resource acquisition and respiration and growth, while there is a hump-shaped relationship between resource acquisition and CUE. The tradeoff between potential for resource acquisition and respiration and growth rates is consistent with the tradeoff between oligotrophic and copiotrophic lifestyles. Oligotrophs are thought to have lower respiration and growth and tend to dominate in nutrient-poor environments (Koch, 2001). Thus, they would be more efficient and invest more assimilated C towards enzyme production. In contrast, copiotrophs have relatively high respiration and growth, where they invest most of their assimilated C towards maintenance of cells, rather than investment in enzymes to utilize more recalcitrant substrates.

We found that CUE and investment in resource allocation have a hump-shaped relationship rather than a consistent negative relationship. This is in contrast with other studies, such as Malik *et al.* (2019), who observed a trend where more efficient microbial communities had less potential for resource acquisition, and communities with higher enzyme regeneration costs had a less efficient physiology. Our study showed that CUE and investment in resource acquisition have a positive relationship (e.g. for the Ascomycota and Mucoromycotina), where

investing energy into enzyme production is beneficial for efficiency. However, this positive relationship has a threshold, where at a certain point the relationship becomes negative (e.g. for the Basidiomycota) and investing too much energy into enzyme production becomes detrimental to efficiency. One notable difference in our study that may explain this dissimilarity is that our experimental conditions were meant to be optimal, so isolates were not C-limited. Thus, for many of the isolates, there may not have been incentive to produce enzymes, making their potential for resource acquisition lower than what might be seen in a soil environment (Schimel and Weintraub 2003). So, our experimental conditions may not have been representative of natural resource acquisition-efficiency demands. Additionally, the observation that some isolates produced enzymes similarly among all environmental treatments suggests that some species undergo constitutive enzyme production. For example, Basidiomycota species *G. subnudus* and *G. dryophilus* had the same levels of NAG activity under the low and high N treatments, suggesting that elevated N will not induce these species to stop producing N-acquiring enzymes. Those that produce enzymes constitutively would be less fit in a resource-poor environment because they would not reallocate assimilated C toward cell maintenance (Allison and Vitousek 2005; Burns *et al.*, 2013). Perhaps if our isolates were grown in more C-limited or complex media, we would have found a stronger tradeoff between CUE and resource acquisition similar to previous studies.

We conclude that there are significant environmental and physiological controls over microbial CUE and that CUE is similar and conserved among differing phylogenetic groups of fungi. In terms of environmental controls over phylogenetic- and species-level CUE, we show that warming had a consistent negative effect, at least in the short-term. On the other hand,

increased N had species-specific effects on CUE that may have important implications at the community-scale. Additionally, we show that when looking across fungal isolates, there is a negative relationship between growth and CUE, which is consistent with the tradeoff we see in soil communities. However, the positive relationship between growth and CUE within phylogenetic groups of fungi clearly demonstrates that this overall negative relationship is likely primarily driven by shifts in community composition and function. It is quite novel that this relationship was accurately represented by the physiology of a small set of fungal isolates, given the complexity of microbial communities. Lastly, we show that there is a tradeoff between investment in resource acquisition and respiration and growth, which further illustrates the unique growth strategies that exist among differing groups of fungi. These results suggest that phylogenetically related fungi have similar and predictable responses to warming and N deposition, and that there are tradeoffs between physiological traits that shape these different growth strategies. These results will provide insight into which ecological classifications (i.e. r- versus K, CSR, YAS) might be most logical to represent microbial function as in C-cycling models.

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TABLES AND FIGURES

Table 1. A factorial ANOVA for specific respiration rate (R_{mass}), specific growth rate (μ), and CUE for the factors temperature (T; 15ºC and 25ºC), nitrogen availability (N; 123:1 and 20:1 C:N), phylum (PHY), and significant interactions. Significant results are highlighted in bold. Insignificant results for combinations are either indicated by dashes or not reported (T * N, N * PHY, and T * N * PHY). Respiration rates and growth rates were square-root transformed to meet assumptions of normality.

	R_{mass}		μ		CUE	
Source	F	P	F	P	F	P
T	27.75	**< 0.0001**	6.53	**0.012**	38.82	**<0.0001**
N	0.30	0.585	0.01	0.947	0.38	0.542
PHY	387.05	**<0.0001**	419.06	**<0.0001**	23.40	**<0.0001**
T * PHY	3.46	**0.035**	---	---	---	---

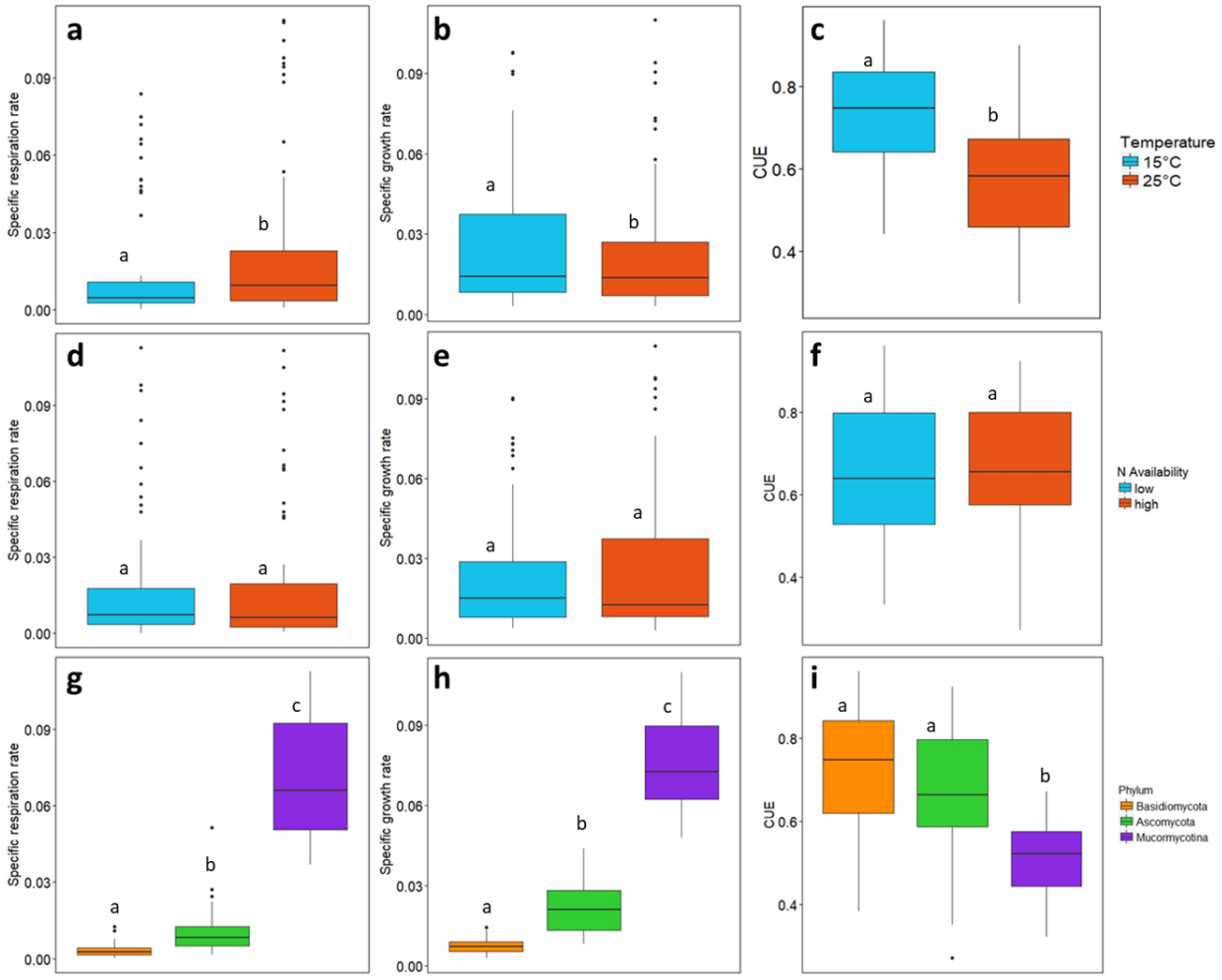

Figure 1. Biomass-specific respiration rates (R_{mass}), biomass-specific growth rates (μ), and CUE for the factors temperature (a-c; 15ºC and 25ºC), N availability (d-f; 123:1 and 20:1 C:N), and phylum (g-i). Respiration rate is expressed as μg CO_2-C hr^{-1} μg biomass-C^{-1}. Growth rate is expressed as μg biomass-C hr^{-1} μg biomass-C^{-1}. CUE is expressed as the ratio of μ to μ plus R_{mass}. Values are pooled across all species. Lines in the boxplot represent the mean. Different letters indicate significant differences as determined by *post hoc* Tukey's HSD test.

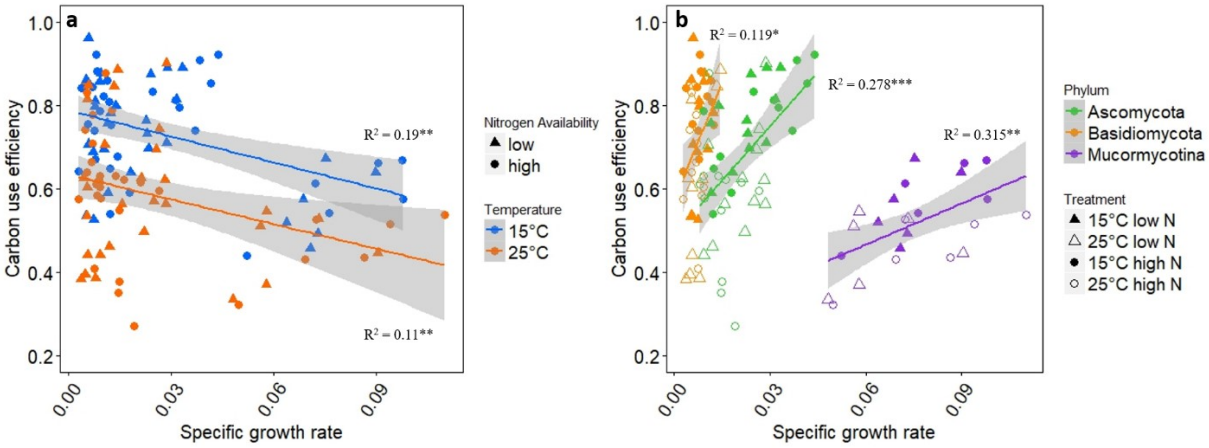

Figure 2. Relationship between growth rate and CUE (a) grouped by temperature or (b) grouped by phyla. Specific growth rate is expressed as μg biomass-C hr^{-1} μg biomass-C^{-1}. CUE is expressed as the ratio of μ to μ plus R_{mass}. The grey bands indicate 95% confidence intervals of the linear regressions. *** represents significance at $P < 0.0001$, ** represents significance at $P < 0.01$, and * represents significance at $P < 0.05$.

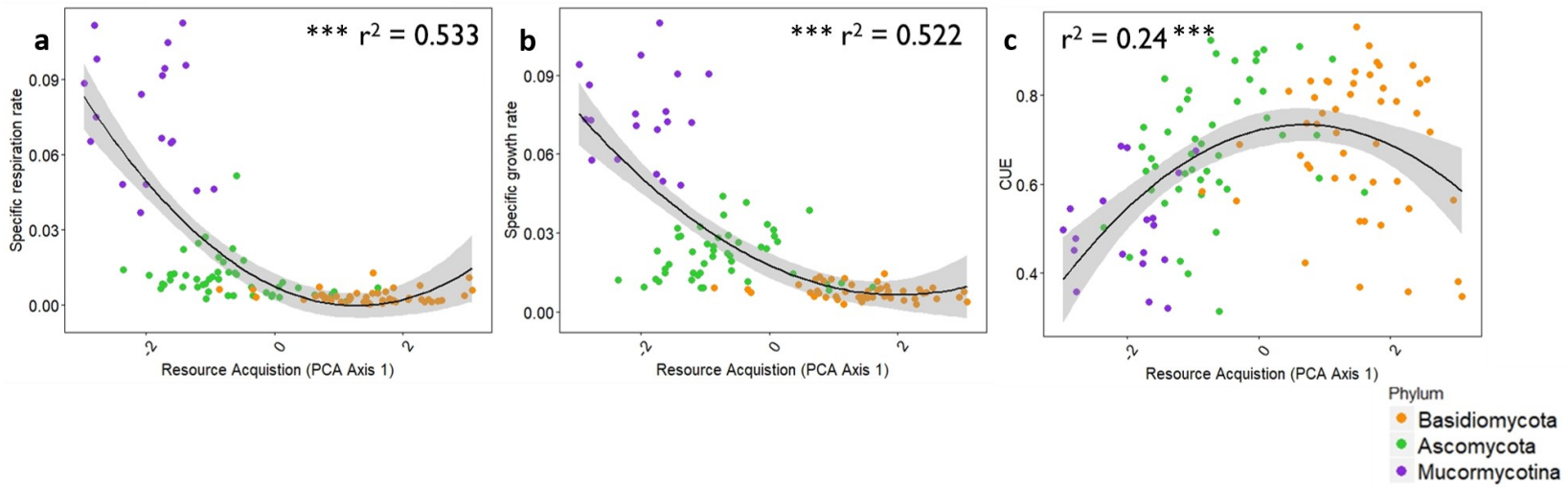

Figure 3. Relationship between resource acquisition and (a) respiration rate, (b) growth rate, or (c) CUE. Biomass-specific respiration rate is expressed as μg CO_2-C hr^{-1} μg biomass-C^{-1}. Biomass-specific growth rate is expressed as μg biomass-C hr^{-1} μg biomass-C^{-1}. CUE is expressed as the ratio of μ to μ plus R_{mass}. Resource acquisition is a synthetic axis created from PCA axis 1 of enzyme activities, where all of the PC scores have been multiplied by -1 so that a low value represents lower composite enzyme activity, and a high value represents higher composite enzyme activity (Fig. S5). Phyla are grouped by color. The grey bands indicate 95% confidence intervals of the polynomial regressions. *** represents significance at $P < 0.0001$.

APPENDEX A: SUPPLEMENTARY TABLES AND FIGURES

Table S1. Factorial ANOVAs of each phylum for biomass-specific respiration rate (R_{mass}), biomass-specific growth rate (μ), and CUE for the factors temperature (T; 15ºC and 25ºC), nitrogen availability (N; 123:1 and 20:1 C:N), species (SP), and significant interactions. Significant results are highlighted in bold. Insignificant results for combinations are indicated by dashes. Respiration rates and growth rates were square-root transformed to meet assumptions of normality.

	Basidiomycota						Ascomycota						Mucoromycotina					
	R_{mass}		μ		CUE		R_{mass}		μ		CUE		R_{mass}		μ		CUE	
Source	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
T	12.4	**0.001**	105.85	**<0.0001**	24.93	**<0.0001**	127.77	**<0.0001**	9.33	**0.0047**	61.42	**<0.0001**	1.25	0.28	0.77	0.39	35.76	**<0.0001**
N	3.06	0.09	1.47	0.236	4.24	**0.048**	3.52	0.07	1.01	0.32	1.88	0.181	1.75	0.21	3	0.11	0.61	0.45
SP	14.39	**<0.0001**	14.53	**<0.0001**	13.12	**<0.0001**	26.89	**<0.0001**	4.56	**0.0095**	18.15	**<0.0001**	0.47	0.51	0.04	0.85	14.1	**0.002**
T * N	---	---	5.12	**0.031**	---	---	4.29	**0.047**	5.35	**0.028**	---	---	---	---	---	---	---	---
T * SP	---	---	8.7	**0.0003**	6.84	**0.001**	3.61	**0.024**	3.74	**0.021**	---	---	---	---	---	---	11.26	**0.0047**
N * SP	---	---	10.57	**0.0001**	8.01	**0.0005**	---	---	---	---	8.08	**0.0004**	---	---	---	---	0.25	**0.002**
T * N * SP	4.13	**0.015**	---	---	3.52	**0.027**	---	---	---	---	---	---	---	---	---	---	12.32	**0.0035**

Table S2. Percent C, percent N, and C:N of dried biomass of each species grown under a low N treatment (123:1) or a high N treatment (20:1).

	C (%)		N (%)		C:N	
Species	*121:1*	*20:1*	*121:1*	*20:1*	*121:1*	*20:1*
Mucor racemosus	43.71	43.84	7.88	7.93	5.55	5.53
Mucor mucedo	43.11	43.69	8.58	8.65	5.02	5.05
Phacidium lacerum	42.34	42.68	2.67	3.03	15.83	14.11
Cylindrium elongatum	45.65	38.53	8.26	7.67	5.53	5.02
Trichoderma koningii	46.97	47.08	3.94	3.84	11.91	12.26
Helotiales sp.	44.55	44.86	7.43	6.90	6.00	6.50
Gymnopus subnudus	43.66	43.97	4.68	5.49	9.32	8.01
Gymnopus dryophilus	42.07	43.39	5.09	6.28	8.27	6.91
Panellus stipticus	43.62	44.35	5.99	7.32	7.29	6.06
Gymnopus sp.	44.63	43.42	4.88	5.07	9.14	8.58

Table S3. Summary of each phylogenetic group's relative growth rate, decomposition potential, and expected and actual CUE response to warming and elevated N. Arrows represent a decrease or increase in average CUE across species within each group with warming or elevated N. Dashes represent no response in average CUE.

Phylum		Growth rate	Decomposition potential	Expected CUE response to warming	Actual CUE response to warming	Expected CUE response to elevated N	Actual CUE response to elevated N
Basidiomycota		low	high	↓	↓	↑	↑
Ascomycota		intermediate	intermediate	↓	↓	↑	–
Mucoromycotina		high	low	↓	↓	↑	–

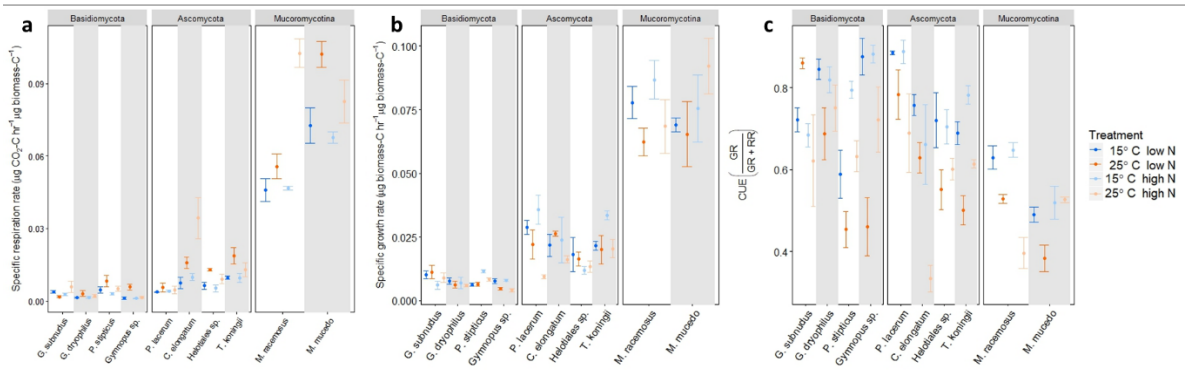

Figure S1. Biomass-specific respiration rate (a; R_{mass}), biomass-specific growth rate (b; µ), and CUE (c) for ten fungal species grown under each of the four treatment combinations. Species are grouped according to phylogenetic relationships. Specific respiration rate is expressed as µg CO_2-C hr^{-1} µg biomass-C^{-1}. Specific growth rate is expressed as µg biomass-C hr^{-1} µg biomass-C^{-1}. CUE is expressed as the ratio of µ to µ plus R_{mass}. Error bars represent standard error.

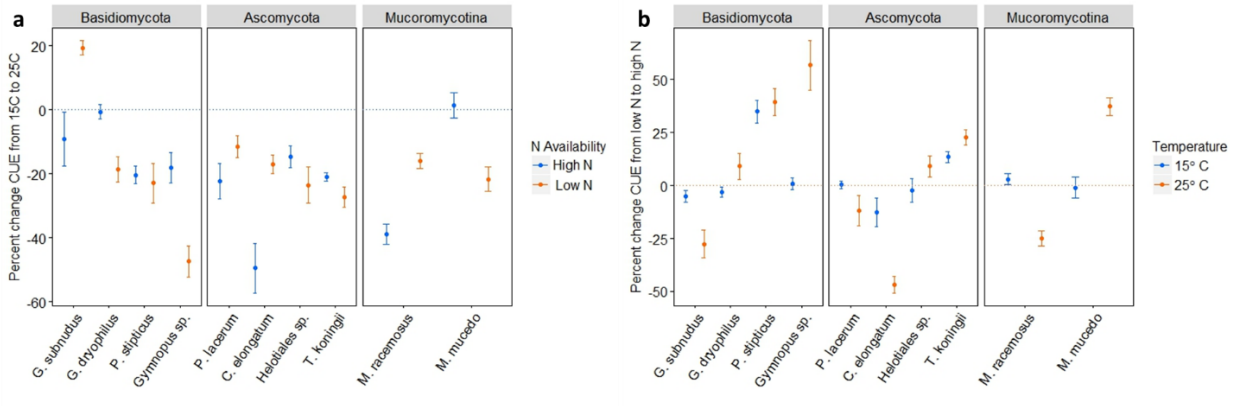

Figure S2. Percent change in CUE from (a) 15ºC to 25ºC, and from (b) low N availability (123:1) to high N availability (20:1) for each species. Species are grouped according to phylogenetic relationships. Dashed line at 0 represents no change in CUE. Error bars represent standard error.

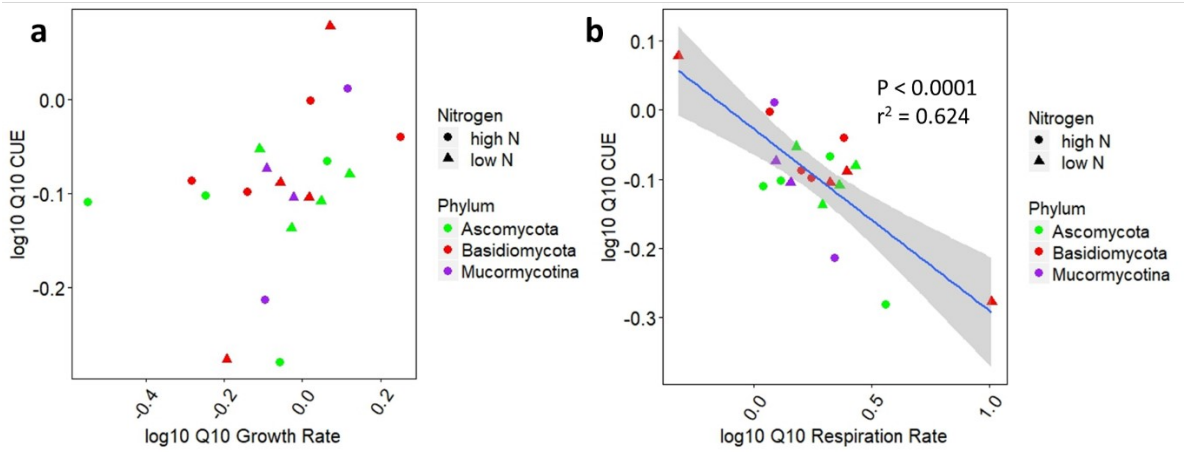

Figure S3. Correlations of Q_{10} CUE versus (a) Q_{10} biomass-specific growth rates, and (b) Q_{10} biomass-specific respiration rates. Q_{10} values were log10 transformed so negative responses to temperature are represented accordingly. The legend indicates N treatment (123:1 and 20:1) and phylogenetic groupings. The grey band (b) indicates 95% confidence intervals of the regression.

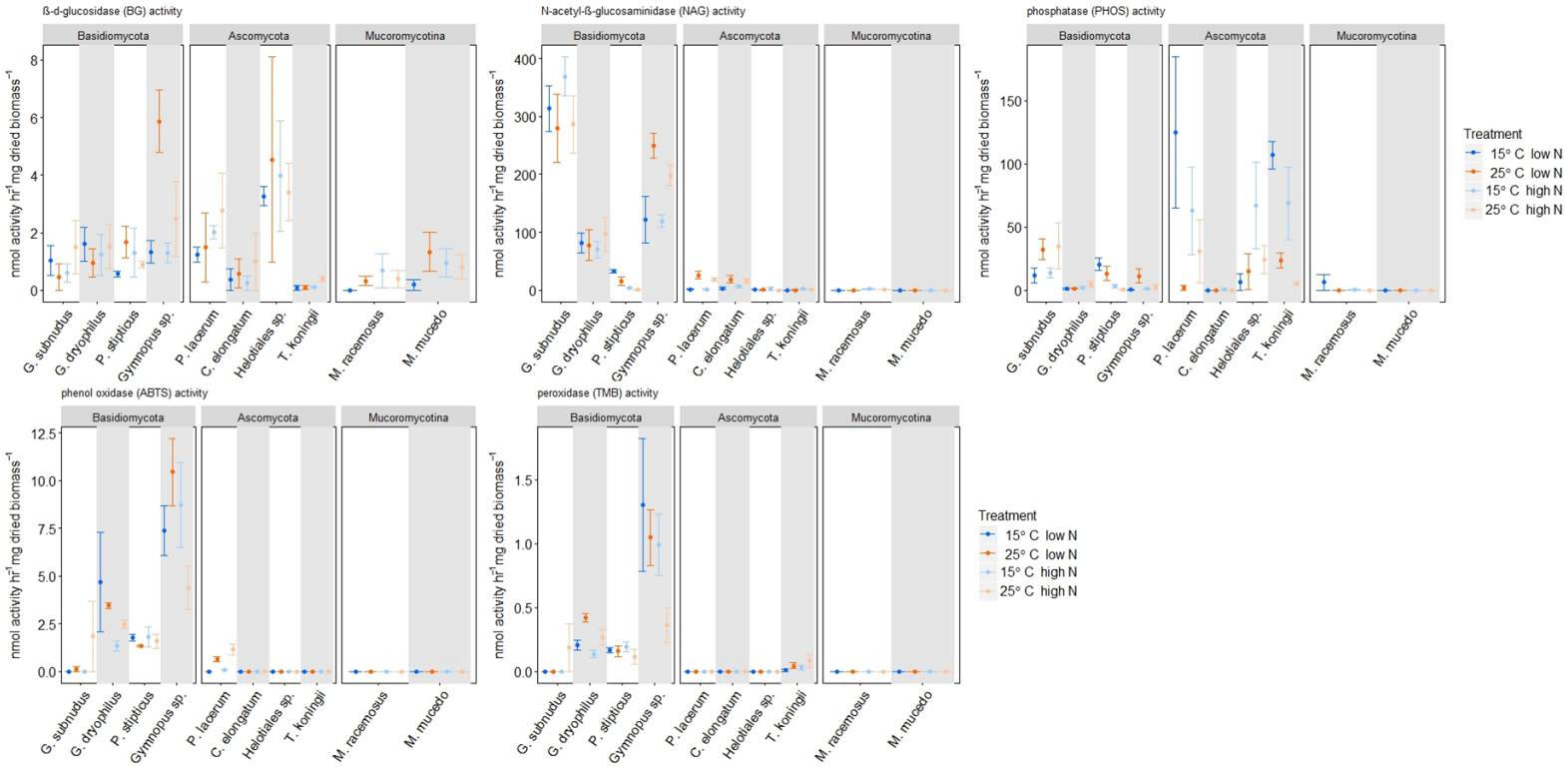

Figure S4. Enzyme activities (BG, NAG, PHOS, ABTS, TMB) for ten fungal species grown under each of the four treatment combinations. Species are grouped according to phylogenetic relationships. Enzyme activity is expressed as nmol activity hr^{-1} mg $biomass^{-1}$. Error bars represent standard error.

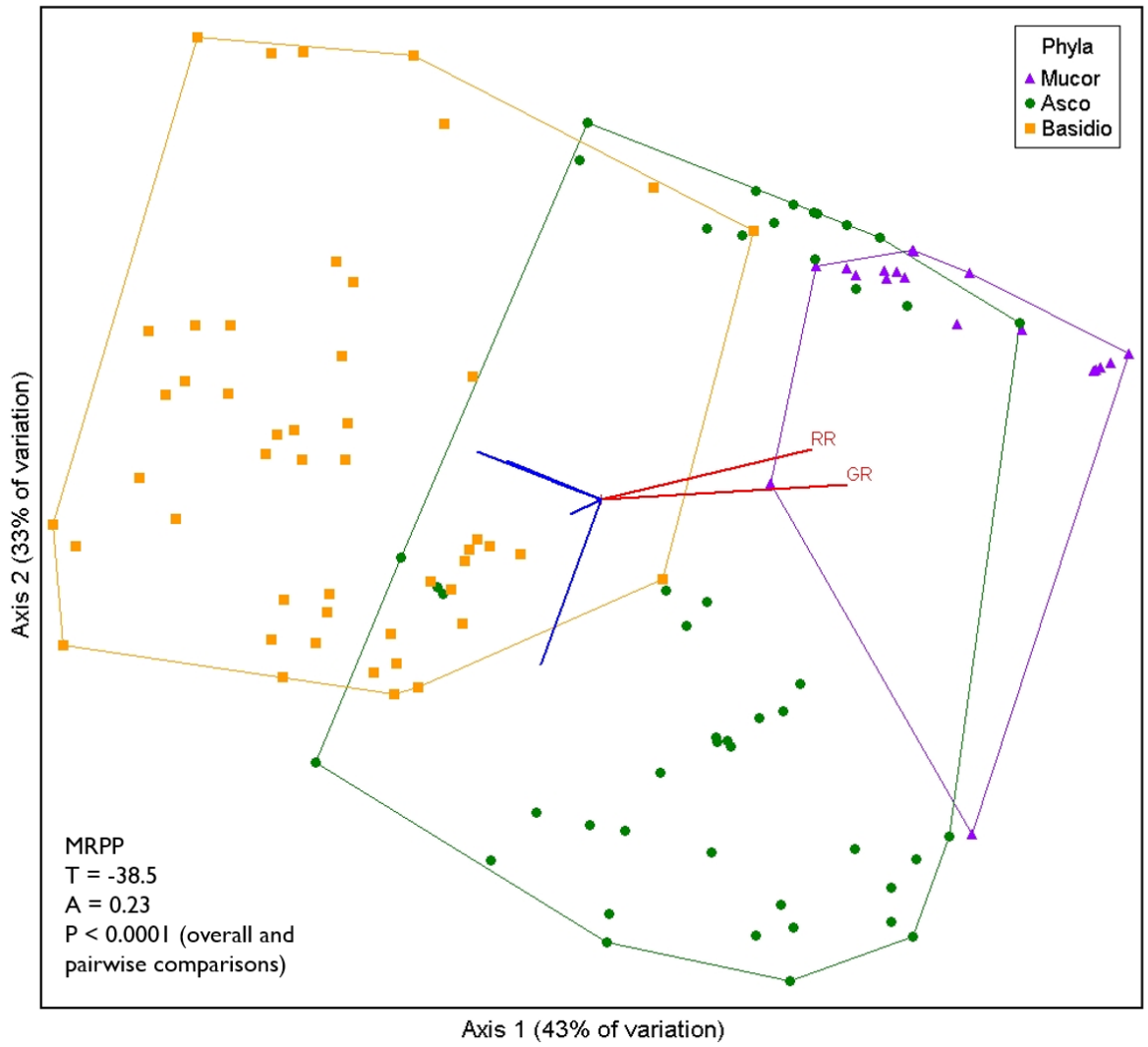

Figure S5. Principal components analysis (PCA) of enzyme activity profiles (grouped by phyla), with MRPP results. The RR biplot vector represents biomass-specific respiration rate and the GR biplot vector represents biomass-specific growth rate. The blue biplot vectors represent the five enzymes' correlation with each PC axis.

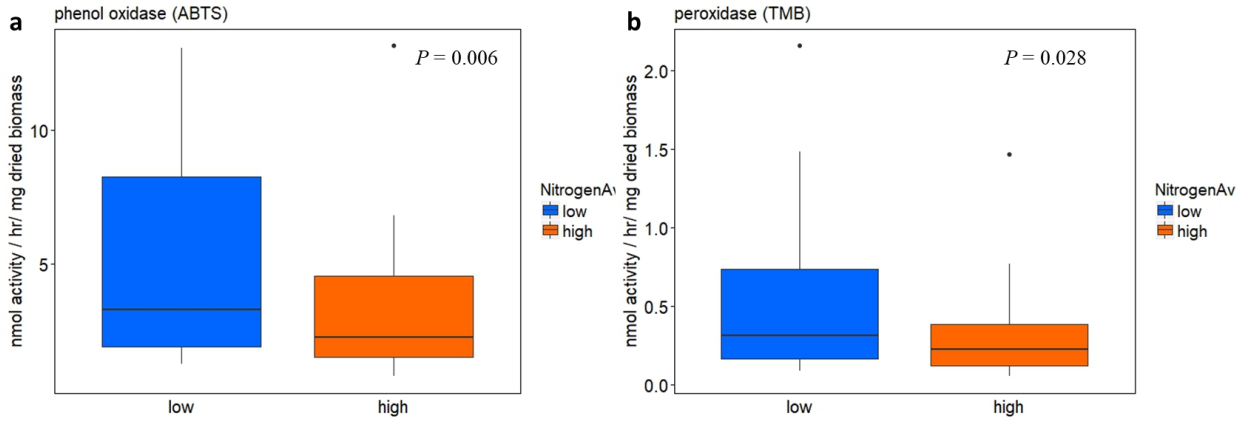

Figure S6. Phenol oxidase (ABTS; a) and peroxidase (TMB; b) activity at low and high N availability (123:1 and 20:1) for three fungal isolates known to be lignin decomposers (*G. dryophilus, P. stipticus, Gymnopus sp.*). Lines in the box plot represents the mean.

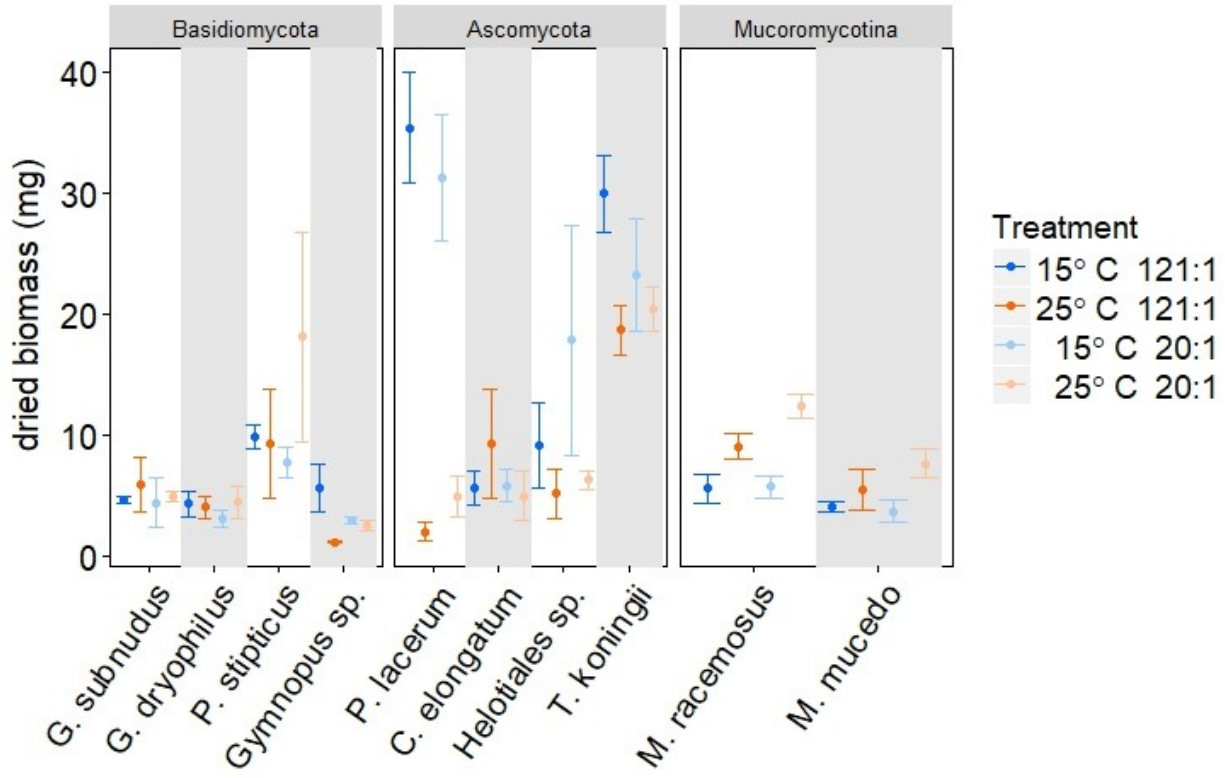

Figure S7. Dried biomass (mg) of one timepoint during log-phase growth for each species x treatment combination. Biomass should only be compared within each species because log-phase growth may have occurred during different timepoints across species. Species are grouped by phylogenetic relationships. Error bars represent standard error.